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This proposal is intended to explore novel strategies that target microtubules that are relevant to breast cancer. Prior studies by the P.I. have led to the cloning of a gene designated Op18, which is expressed at high levels in breast cancer. Op18 plays a regulatory role in microtubule transition through binding to tubulin dimers. The identifiaction of Op18 as a regulator of microtubules is of significance in view of the importance of microtubules and their regulated growth or shrinkage in a number of essential cellular processes including progression through the cell cycle, maintenance of cell shape, and intracellular transport and in view of the relevance of microtubules as targets of breast cancer therapy. This project has two objectives. One is to test the hypothesis that Op18 levels as well as its phosphorylation status affect the ability of breast cancer cells to progress through the cell cycle and to proliferate. The second objective is to test the hypothesis that Op18 exerts a regulatory role on microtubule assembly that is dependent on both Op18 level and phosphorylation status. In the past year we have obtained evidence that Op18 microtubule destabilizing activity is dependent on phosphorylation. We have also obtained evidence of substantial variabiity in both Op18 and of tubulin contents in breast tumors and cell lines. Both in vitro and transgenic mouse models have been developed that should allow in the coming year assessment of the role of Op18 levels in proliferation and of the growth properties of transfected breast cancer cells and of their response to taxol.

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TBLE OF CONTENTS

INTRODUCTION

The microtubule system has emerged as an important target of chemotherapeutic intervention in breast cancer (1-4). This proposal is intended to explore novel strategies that target microtubules that are relevant to breast cancer. The dynamic turnover of microtubules within the cell is essential for a number of cellular processes including progression through the different phases of the cell cycle, intracellular transport and maintenance of cell shape. Disruption of microtubule turnover results in inability of cells to progress through the cell cycle. In particular, the drug taxol has an antiproliferative effect due to its stabilization of microtubules. An understanding of the regulation of microtubule transitions is highly relevant to the targeting of microtubules for chemotherapeutic intervention. Tubulin is an integral component of microtubules. It occurs primarily as soluble heterodimers consisiting primarily of α- and β-tubulin isoforms or as assembled tubulin polymers that form microtubules (5)). Post-tanslational modification and differential gene expresion contribute to tubulin heterogeneity between tissues. This heterogeneity is reflected by the varied migration pattern of tubulin isoforms in 2-D gels prepared from different tissues. A number of enzymes can induce rapid changes in tubulin structure, particularly at the highly variable carboxy terminal region of the molecule (6). In vertebrates, expression of tubulin isotypes is complex. Some occur at high level in certain tissues and their pattern of expression changes during development (5).

Prior studies by the P.I. have led to the cloning of a gene designated Op18, which is expressed at high levels in a number of tumor types including breast cancer (7-10). Subsequent studies have shown that this gene plays a critical role, through phosphorylation of its protein product, in progression through the cell cycle (11-15). Mutation of this gene at Ser residues, which are normally phosphorylated during cell cycle progression, results in mitotic arrest in the G2/M phase (16). The Op18 gene has been demonstrated to play a regulatory role in microtubule transition through binding of the protein it encodes to tubulin dimers (17,18). Op18 protein was found to destabilize microtubules, an effect which is diametrically opposite to that of taxol (19,20). A compelling case can therefore be made for: 1) understanding the role Op18 may play in regulating cell cycle transition in breast cancer cells; 2) examining the effect of disrupting Op18, through mutation, on breast cancer cell growth; and 3) examining the effect of manipulating Op18 expression on response of breast cancer cells to taxol.

The identification of Op18 as a regulator of microtubules is of substantial significance in view of the large body of evidence that points to the importance of microtubules and their regulated growth or shrinkage (microtubule dynamics) in a number of essential cellular processes include progression through the cell cycle, maintenance of cell shape, and intracellular transport. The dynamic turnover of microtubules within the cell contributes to their formation of different arrays including the radial arrangement in proliferating interphase cells, the mitotic spindle in dividing cells, and the staggered linear array in axons. The regulation of microtubule transitions during the cell cycle are likely regulated by kinases that also phosphorylate Op18 (19,20). Addition of Cdc2 kinase to cell extracts is sufficient to alter microtubule lengths from those typical of interphase to mitotic levels kinase microtubules are essential for a number of cellular processes, Op18 therefore through its regulation of microtubules, plays an important role in cell functions that are dependent on microtubules.

BODY

EXPERIMENTAL METHODS

This project has two interrelated objectives:

1- One is to test the hypothesis that Op18 levels as well as its phosphorylation status affect the ability of breast cancer cells to progress through the cell cycle and to proliferate. The testing of this hypothesis is accomplished by transfection of different constructs containing: Op18 in the sense or antisense orientation; vector alone; or Op18 mutated at phosphoserine sites and examining the effect of manipulated expression of Op18 on transfected cells.

2- The second objective of this project is to test the hypothesis that Op18 exerts a regulatory role in microtubule assembly that is dependent on both Op18 level and phosphorylation status. The testing of this hypothesis is accomplished by examining the effect of Op18 levels and mutations on microtubule assembly and by determining the sensitivity of breast cancer cells transfected with different Op18 constructs to the microtubule stabilizing drug taxol.

The major experiments proposed to achieve project objectives, particularly in the first year are listed below.

Manipulations of Op18 expression

Desired sequence changes in Op18 had been made by a PCR-based strategy prior to the start of the project, using synthetic oligonucleotides to introduce a point mutation (substitution of Ala for Ser at phosphorylation sites), because of the efficiency of this approach. To reduce the potential for polymerase generated error (and the amount of sequencing required to rule it out), mutations were introduced by amplifying only small fragments. As part of this project we have planned transfection studies. Our planned approach was to clone Op18 cDNA into pBS-SK$^+$ vector in the *Xho*I site. Op18-pBS-SK$^+$ is then cut with *Not*I which cleaves next to the initiation codon of Op18 cDNA. *Eco*RI-*Not*I adaptors (AAT TCG CGG CCG C) are added to the 5' end of the coding region which would provide a better ribosome-binding site for efficient translation. After digestion with MScI, either *Bam*HI or *Hin*dIII linkers are added. Expression vector pCEP4 (Invitrogen) is digested with *Not*I and *Bam*HI or *Not*I and *Hin*dIII, and ligated with properly digested Op18 cDNA fragments which have been modified with *Eco*RI-*Not*I adaptor on the 5' end and *Bam*HI or *Hin*dIII linkers on the 3' end. Op18 cDNA is cloned through *Not*I-*Bam*HI and *Not*I-*Hin*dIII ligation, respectively. Both sense constructs and constructs containing either one of the mutations described and pCEP4 vector alone are transfected into cells by electroporation. The transfectants are selected with hygromycin.

Phosphorylation state of Op18 bound to tubulin

Prior studies have shown that Op18 physically interacts with tubulin dimers, in part on the basis of co-fractionation by gel filtration. There are at least two alternative scenarios that are compatible with this finding, that are fairly readily testable: One is that the proportion of phosphorylated Op18 forms that co-fractionate with tubulin dimers is similar to the overall proportions of phosphorylated Op18 in the cell. Another is that Op18 that co-fractionates with tubulin exhibits a different phosphorylation profile than whole cell Op18. The prevailing scenario can be determined by 2-D PAGE analysis of Op18 that co-fractionates with tubulin dimers, an approach that is fairly readily accomplished in our laboratory. The location of tubulin subunits in 2-D gels is well characterized in our laboratory, as is of course that of the different Op18 isoforms. These studies will also investigate whether mutated Op18 fails to co-localize with tubulin or alternatively, it co-localizes to a greater extent than normal Op18.

A variation along this line of investigations is to mix preparations of tubulin and normal or mutant Op18 in an unphosphorylated or phosphorylated state and analyze gel filtration fractions by 2-D PAGE, for physical interaction between Op18 forms and tubulin. Yet another variation, in view of the dominant negative effect of some Op18 mutants is to determine if certain Op18 forms prevent interaction of others with tubulin, when mixed together.

RESULTS AND DISCUSSION

Effect of Op18 phosphorylation on interaction with tubulin

We have undertaken experiments in which preparations of tubulin and Op18 in an unphosphorylated or phosphorylated state were allowed to interact and gel filtration fractions were analyzed for evidence of physical interaction between Op18 forms and tubulin.

Tubulin was prepared as previously described and recombinant Op18 was utilized after dephosphorylation with alkaline phosphatase or after phosphorylation with a number of kinases known to phosphorylate Op18 including protein kinase A, cdc2 kinase and casein kinase. The stoichiometry of the interaction was determined by mixing of phosphorylated or unphosphorylated Op18 and purified tubulin in different ratios. The data showed that unphosphorylated Op18 was predominently responsible for tubulin depolymerizing activity, while phosphorylation of Op18 with different kinases impaired tubulin destabilizing activity. One aspect that was not appreciated at the start of this project is the extensive nature of variability in tubulin primary structure. There is considerable tubulin heterogeneity resulting from a large tubulin gene family encoding numerous isotypic forms, and from numerous post-translational modifications of tubulin (6) . Some of the modifications include acetylation, phosphorylation, tyrosination, polyglutamylation, polyglycylation These post-translational modifications result from the action of a number of specific modification-enzymes. Although the significance of tubulin post-translational modifications is not well understood, it is likely that they impact on the role of microtubules in the cell. It follows therefore that the effect of phosphorylation of Op18 on its interaction with tubulin may depend of the specific tubulin isotype as well as on any post-translational modifications of this isotype. Given the enourmous variety of tubulin isoforms that occur in cells, the testing of the different forms is a daunting process and a strategy has been devised to tackle this problem, as indicated below.

Op18 and tubulin expression in breast cancer

We have investigated expression of Op18 at the protein level in 32 primary breast adenocarcinomas, to date, and in a number of cell lines. A cell line, SUM-102PT, developed by our collaborator, Dr. Steve Ethier, from a specimen obtained from a patient diagnosed with intraductal carcinoma of the breast with micro-invasion has been primarily utilized for our studies. The cell line was isolated using a growth factor deficient medium that does not support growth of normal mammary epithelial cells. SUM-102PT cells have been proliferating in serum-free medium and are responsive to, but do not require, epidermal growth factor and progesterone.. SUM-102PT cells are near-diploid, and karyotype studies indicate that they do not possess abnormalities in chromosome 1, to which the Op18 gene has been mapped. Analysis of tumor and cell line protein constituents by 2-D PAGE showed that Op18 is expressed but at avariable levels in all primary tumors and is prominently expressed in phosphorylated and unphosphorylated forms in SUM-102PT. In approximately half of the primary tumors, Op18 occurred as a major protein constituent, as observed in a number of solid tumors including small cell lung cancer, GI tumors and childhood tumors. Op18 occurred at more moderate levels in the remainder of the tumors. Studies of changes in Op18 in cultured cell lines indicated that there was no effect of cell density on Op18 levels and phosphorylation. Additionally hormone dependent cell lines did not alter their pattern of expression of Op18 as a function of hormone treatment. Analysis of breast cancer tumors and cell lines by 2-D PAGE also revealed a variable amount of known tubulin polypeptide alpha and beta forms. This was particularly more accentuated for tubulin beta. The identity of tubulin beta was determined by N-terminal sequencing as well as by mass spectrometric analysis. Interestingly however, there appeared to be variabilty in the representation of peptides derived from different tubulin beta isoforms, suggesting differential expression of beta tubulin among different tumors. These findings raise the interesting possibility that certain tubulin isotypes or isoforms may be particularly sensitive to the effect of Op18. A major difficulty in prusuing this hypothesis further is the apparent difficulty in producing tubulin isotypes in recombinant form, which account for the continued reliance on tubulin purified from animal tissue. Our strategy for pursuing these investigations will have to remain at a correlational level, namely by correlating tubulin isoforms in particular tumors and cell lines with the level and phosphorylation status of Op18. Fortunately in this regard the mass spectrometric analysis of proteins and peptides has become more easily attainable so that a greater number of tumors and cell lines can be investigated for teir content of different tubulin isoforms.

Manipulations of Op18 expression

Manipulation of the levels of normal Op18 was done using a constitutively active promoter for greater efficiency. Both sense and antisense Op18 constructs and pCEP4 vector alone were transfected into cells by electroporation and the transfectants selected with hygromycin. We observed early on that the transfectant containing sense Op18 exhibited a higher proliferation rate and increased formation of transformed foci. For verification of the levels of Op18, sufficient cells were cultured to determine by 2-D PAGE that indeed Op18 levels were manipulated. However over the period of time that it took to achieve sufficient cell number the phenotype appeared to reverse itself. This was evidently due to loss of transfected Op18. This unfortunate situation occurred again a second time making us change our strategy to manipulate Op18 levels. The strategy

since has consisted of reliance on an inducible retroviral system developed by our colleague and collaborator Eric Radany here at the Unviversity of Michigan. The constructs have been reengineered and transfections have been recently initiated.

A second approach has also been utilized to determine the effect of manipulated expression of Op18. This approach relies on the development of transgenic models. Op18 wild type construct and an Op18 which has been mutated at all phosphorylation sites have been utlized to develop transgenic models. We have utilized the inducible Tet system for activated expression of the transgenes (21). At the present time, progress has been made up to the birth of F1 mice carrying either the wild type or the mutated Op18 gene as well as carrying the Tet transgene. Some sixty positive mice are currently available. We are in the process of inducing expression in a subset of these mice to determine the efficiency of expression. In subsequent generations, expression will be induced at different stages during the development and post-natally to determine the effect on specific tissues.

RECOMMENDATIONS IN RELATION TO THE STATEMENT OF WORK IN THE PROPOSAL

The goals of this project remain essentially unchanged. In the coming year, we plan to investigate the effect of manipulating Op18 levels and of introducing mutations in Op18 on the transformed properties of transfected cells using the new constructs. Transfected and control cells will be injected into 6-8-week-old female CB17-Scid/Scid mice. Mice will be monitored on a daily basis and will be sacrificed when moribund. At necropsy, mice will be examined for the presence of gross tumors. Tissues will be analyzed microscopically for presence of tumor infiltrates. Proteins and DNA from different tissues will be investigated for Op18 levels by 2-D PAGE and for the presence of human genes (HLA-DQ sequences) by Southern.

We would like to determine the effect of Op18 gene manipulations on sensitivity of the SUM-102PT breast cancer line to taxol, in the Scid mouse. The effect of taxol on cell transfectants will be first analyzed *in vitro*, followed by analysis of sensitivity of transfected cells in the Scid mouse. In one experiment, we will examine the effect of taxol on mice infused with cells containing Op18 sense, Op18 antisense or vector alone

Additionally the effect of inducing expression of wild type and of mutant Op18 will be examined in a transgenic mouse model. Of particular interest is the effect of Op18 on microtubules and of the particular senstivity of some tissues compared to others.

REFERENCES

(1) Rowinsky, E., Cazenave, L., Donehower, R. (1990) Taxol: A novel investigational antimicrotubule agent. *J. Natl. Cancer Inst.* **82,** 1247-1259.

(2) Derry, W., Wilson, L., Jordan, M. (1995) Substoichiometric binding of taxol suppresses microtubule dynamics. *Biochem.* **34,** 2203-2211.

(3) Rowinsky, E., Donehower, R. (1993) The clinical pharmacology of paclitaxel (taxol). *Semin. Oncol.* **20,** 16-25.

(4) Arbuck, S., Canetta, R., Onetto, N., Chrishan, M. (1993) Current dosage and schedule issues in the development of paclitaxel (Taxol). *Semin. Oncol.* **20,** 31-37

(5) Ludena, R. (1998) Multiple forms of tubulin:different products and covalent gene modifications. Int Rev Cytology **178**. 207-275.

(6) Macrae, H., Tubulin post-translational modifications. Eur J Bicohem **244**, 265-278.

(7) Nichols, D. (1988) Identification of a polypeptide associated with the malignant phenotype in acute leukemia. *J. Biol. Chem.* **263,** 12813-12815.

(8) Zhu, X. X., Kozarsky, K., Strahler, J. R., Eckerskorn, C., Lottspeich, F., Melhem, R., Lowe, J., Fox, D. A., Hanash, S. M., Atweh, G. F. (1989) Molecular cloning of a novel human leukemia associated gene: Evidence of conservation in animal species. *J. Biol. Chem.* **264,** 14556-14560.

(9) Melhem, R. F., Zhu, X. X., Hailat, N., Strahler, J., Hanash, S. M. (1991) Characterization of the gene for a proliferation related phosphoprotein (Op18) expressed in high amounts in acute leukemia. *J. Biol. Chem.* **266,** 17747-17753.

(10) Strahler, J. R., Hailat, N., Lamb, B. J., Rogers, K. P., Underhill, J., Melhem, R. F., Keim, D. R., Zhu, X.-X., Kuick, R. D., Fox, D. A., Hanash, S. M. (1992) Activation of peripheral blood lymphocytes through the T cell receptor induces rapid phosphorylation of Op18. *J. Immunol.* **149,** 1191-1198.

(11) Leighton, I., Curmi, P., Campbell, D., Cohen, P., Sobel, A. (1993) The phosphorylation of stathmin by MAP kinase. *Molecular and Cellular Biochemistry* **127/128,** 151-156.

(12) Marklund, U., Brattsand, G., Shingler, V., Gullberg, M. (1993) Serine 25 of oncoprotein 18 is a major cytosolic target for the mitogen-activated protein kinase. *J. Biol. Chem.* **268,** 15039-15047.

(13) Beretta, L., Dobransky, T., Sobel, A. (1993) Multiple phosphorylation of stathmin. *J. Biol. Chem.* **268,** 20076-20084.

(14) Luo, X.-N., B, Ferrari, A., Mistry, S., Atweh, G. (1994) Regulation of phosphoprotein p18 in leukemic cells. *J. Biol. Chem.* **269,** 10312-10318.

(15) Melhem, R. F., Strahler, J. R., Hailat, N., Zhu, X. X., Hanash, S. M. (1991) Involvement of Op18 in cell proliferation. *Biochem. Biophys. Res. Commun.* **179,** 1649-1655.

(16) Marklund, U., Osterman, O., Melander, H., Bergh, A., Gullberg, M. (1994) The phenotype of a "cdc2 kinase target site-deficient" mutant of oncoprotein 18 reveals a role of this protein in cell cycle control. *J. Biol. Chem.* **269,** 30626-30635.

(17) Belmont, L., Mitchison, T. (1996) Identification of a protein that interacts with tubulin dimers and increases the catastrophe rate of microtubules. *Cell* **84,** 623-631.

(18) Cassimeris, L. (1993) Regulation of microtubule dynamic instability. *Cell Motil. Cytoskeleton* **26,** 275-281.

(19) Verde, F., Labbe, J., Karsente, E. (1990) Regulation of microtubule dynamics by CDC2 kinase. *Nature* **343,** 233-238.

(20) Gotoh, Y., Nishida, E., Sakai, H. (1991) In vitro effects on microtubule dynamics of MAP kinase. *Nature* **349,** 251-254.

(21) Gossen, M., Bujard, H. (1992) Tight control of gene expression in mammalian cells by tetracycline-responsive promoters. *Proc. Natl. Acad. Sci. USA* **89,** 5547-5551.